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(54) Title: ANTIMICROBIAL DRUG SCREENING USING A RECOMBINANT CELL COMPRISING A RNA–DEPENDENT AMIDOTRANSFERASE GENE

(57) **Abstract**

This invention relates to newly developed methods for discovering antimicrobial compounds using an RAT–based assay system. This invention also relates to compositions of matter useful in carrying out the methods of the invention as well as antimicrobial compounds developed using such methods.

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ANTIMICROBIAL DRUG SCREENING USING A RECOMBINANT CELL COMPRISING A RNA-DEPENDENT AMIDOTRANSFERASE GENE

FIELD OF THE INVENTION

This invention relates to newly developed methods for discovering antimicrobial compounds using a RAT gene-based whole cell assay. It is particularly suited for carrying out antimicrobial compound screening assays in bacterial cells. This invention also relates to compositions of matter useful in carrying out the methods of the invention as well as antimicrobial compounds developed using such methods.

BACKGROUND OF THE INVENTION

Despite the absence of glutaminyl-tRNA synthetase activity in Gram-positive bacteria, and likely all Gram-negative bacteria with the exception of the gamma subdivision of purple bacteria, bacterial cells are clearly able to produce the Gln-tRNA(Gln) required for accurate protein synthesis. The mechanism by which this is achieved involves the formation of Glu-tRNA(Gln) as an intermediate that is produced by the misaminoacylation of tRNA(Gln) by glutamyl-tRNA synthetase (ERS). This reaction would be toxic as it would lead to Gln-tRNA(Gln) starvation and to the synthesis of aberrant proteins and the consequent cessation of bacterial protein synthesis. However, the 'correct' end product, Gln-tRNA(Gln), is formed from Glu-tRNA(Gln) by transfer of an amine group to the ligated glutamate residue. This reaction is catalyzed by a tRNA- and Mg^{2+}/ATP-dependent amidotransferase. (**R**NA-dependent **A**mido**T**ransferase - **RAT**) ; also known as Glu-$tRNA^{Gln}$ amidotransferase or Glu-AdT – Curnow-AW, *et al. PNAS 94*, 11819-11826 (1997). Inhibition of this apparently ubiquitous reaction in Gram-positive organisms, and some Gram-negative organisms, would effectively lead to Gln-tRNA(Gln) starvation and to cell death.

This invention provides methods for exploiting the relationship between RAT and glutamyl-tRNA synthetase to screen for antimicrobial compounds that target either or both of these enzymes, for example by binding to them or affecting their enzymatic pathways. There is a need for methods for screening for novel antimicrobial compounds, such as the screening methods of the invention. Such methods have a present benefit of being useful to screen compounds for antibiotic activity that can play a role in preventing, ameliorating or correcting infections, dysfunctions or diseases, such as bacterial infections.

GLOSSARY

The following definitions are provided to facilitate understanding of certain terms used frequently herein. Certain other definitions are provided elsewhere herein.

"Host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

"Isolated" means altered "by the hand of man" from its natural state, *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotide(s)" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. "Polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other

than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, *PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., *Meth. Enzymol. 182*:626-646 (1990) and Rattan et al., *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

"Variant(s)" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions,

deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to skilled artisans.

SUMMARY OF THE INVENTION

A method of screening for antimicrobial drugs comprising the steps of: providing at least one cell naturally lacking a RAT gene or genes and comprising at least one recombinant glutamyl tRNA synthetase gene and at least one recombinant RAT gene; contacting the cell with at least one candidate compound; and detecting altered metabolism in the cell of the contacting step. Methods are also provided for screening in *Staphylococcus aureus* that uses a wild type glutamyl tRNA synthetase gene.

A method wherein the recombinant glutamyl tRNA synthetase and RAT genes are on an episomal element or integrated into a chromosome of the cell.

A method wherein RAT gene expression level is regulated.

A method wherein a RAT gene is a RAT gene selected from the group consisting of a Gram positive bacteria, a Gram negative bacteria, a streptococcus, *S. pneumoniae*, a staphylococcus, *S. aureus*, enterococci, *Enterococcus faecalis*, *Enterococcus faecium*, a Bacillus, and *Bacillus subtilis*.

A method wherein the glutamyl tRNA synthetase gene is a glutamyl tRNA synthetase gene selected from the group consisting of a Gram positive bacteria, a Gram negative bacteria, a streptococcus, S. pneumoniae, a staphylococcus, S. aureus, enterococci, Enterococcus faecalis, Enterococcus faecium, a Bacillus, and *Bacillus subtilis*.

A method wherein the altered metabolism comprises inhibition of RAT protein activity.

A method wherein the detecting step further comprises detecting, an accumulation, particularly a toxic accumulation, of Glu-tRNA(Gln) or a toxic incorporation of a glutamyl residue for a glutaminyl in a nascent protein chain.

A method wherein the detecting step further comprises detecting cell death or a reduction in growth rate or amount, particularly a substantial reduction in growth rate or amount.

A method wherein the cell possesses a glutaminyl tRNA synthetase or lacks a RAT gene.

A method wherein the cell lacks a glutaminyl tRNA synthetase or possesses a RAT gene, particularly *S. aureus*.

An isolated bacterial cell lacking a RAT gene and comprising at least one recombinant bacterial glutamyl tRNA synthetase gene and at least one recombinant bacterial RAT gene.

An isolated bacterial cell possessing a RAT gene and comprising at least one recombinant or wild type bacterial glutamyl tRNA synthetase gene and at least one recombinant bacterial RAT gene.

A method or composition wherein the glutamyl tRNA synthetase and RAT genes are on episomal element or integrated into a chromosome of the cell.

A host cell wherein the RAT gene expression level is regulated.

A host cell wherein a RAT gene is an *S. aureus* RAT gene.

A host cell wherein the glutamyl tRNA synthetase gene is a *S. aureus* glutamyl tRNA synthetase gene.

A host cell wherein the cell possesses a glutaminyl tRNA synthetase or lacks a RAT gene.

A host cell wherein the cell lacks a glutaminyl tRNA synthetase or possesses a RAT gene.

A method wherein the detecting step further comprises detecting altered translation.

A method wherein the detecting step further comprises detecting altered test protein.

A kit comprising at least one bacterial cell lacking a RAT gene and the cell comprising at least one bacterial glutamyl tRNA synthetase gene and at least one bacterial RAT gene.

A kit comprising a polynucleotide encoding a RAT gene and a polynucleotide encoding a glutamyl tRNA synthetase gene.

A polynucleotide comprising a RAT expressibly linked to an inducible promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 shows a schematic diagram of a preferred embodiment of the invention showing a schematic diagram of the putative mechanism of an embodiment of the invention.

Figure 2 shows a graph demonstrating the putative mechanism of one preferred embodiment of the invention.

Figures 3 shows a schematic diagram of a preferred embodiment of the invention showing a schematic diagram of the putative mechanism of an embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for screening for compounds that lead to or are involved in aborted translation, particularly those that do not inhibit protein synthesis *per se*. Applicants believed that such compounds will have an enormous impact on 'global' protein synthesis, and are therefore predicted to be bactericidal.

The natural promoter of the RAT gene (herein "RAT gene(s)" means a gene encoding any or all of the RAT protein subunits in an enzymatically active or biologically functional RAT protein complex (*e.g.*, a RAT gene in Table 1), and "RAT protein(s)" means any protein encoded by a RAT gene (*e.g.*, a RAT protein in Table 1)) will be replaced with a heterologous, regulatable promoter (*e.g.*, an inducible promoter) in the chromosome of a RAT-expressing bacterial host cell, such as by homologous recombination (in a preferred embodiment insertional mutagenesis is used since, for example, it is more rapid than a double crossover and should give the same phenotype. Such RAT gene constructs comprising a regulatable promoter is referred to as "hybrid RAT." However, by using this method there will be an extra copy of the first stretch of base pairs (e.g., about 300-700 base pairs, preferably about 500 base pairs) of the RAT gene present, still under the control of the native RAT promoter. This will not be sufficient sequence to encode active RAT).

Preferred host cells useful in the invention include, but are not limited to, any bacteria comprising a natural endogenous RAT gene, such as, any Gram positive bacteria, many Gram negative bacteria, and also a member of the genus *Streptococcus,*

Staphylococcus, Bordetella, Corynebacterium, Mycobacterium, Neisseria, Haemophilus, Actinomycetes, Streptomycetes, Nocardia, Enterobacter, Yersinia, Fancisella, Pasturella, Moraxella, Acinetobacter, Erysipelothrix, Branhamella, Actinobacillus, Streptobacillus, Listeria, Calymmatobacterium, Brucella, Bacillus, Clostridium, Treponema, Escherichia, Salmonella, Kleibsiella, Vibrio, Proteus, Erwinia, Borrelia, Leptospira, Spirillum, Campylobacter, Shigella, Legionella, Pseudomonas, Aeromonas, Rickettsia, Chlamydia, Borrelia and *Mycoplasma*, and further including, but not limited to, a member of the species or group, Group A *Streptococcus*, Group B *Streptococcus*, Group C *Streptococcus*, Group D *Streptococcus*, Group G *Streptococcus, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus faecalis, Streptococcus faecium, Streptococcus durans, Neisseria gonorrheae, Neisseria meningitidis, Staphylococcus aureus*, paricularly *Staphylococcus aureus* strain RN4220, *Staphylococcus epidermidis, Corynebacterium diptheriae, Gardnerella vaginalis, Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium ulcerans, Mycobacterium leprae, Actinomyctes israelii, Listeria monocytogenes, Bordetella pertusis, Bordatella parapertusis, Bordetella bronchiseptica, Escherichia coli, Shigella dysenteriae, Haemophilus influenzae, Haemophilus aegyptius, Haemophilus parainfluenzae, Haemophilus ducreyi, Bordetella, Salmonella typhi, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris, Yersinia pestis, Kleibsiella pneumoniae, Serratia marcessens, Serratia liquefaciens, Vibrio cholera, Shigella dysenterii, Shigella flexneri, Pseudomonas aeruginosa, Franscisella tularensis, Brucella abortis, Bacillus anthracis, Bacillus cereus, Clostridium perfringens, Clostridium tetani, Clostridium botulinum, Treponema pallidum, Rickettsia rickettsii* and *Chlamydia trachomitis*, (ii) an archaeon, including but not limited to *Archaebacter*, and (iii) a unicellular or filamentous eukaryote, including but not limited to, a protozoan, a fungus, a member of the genus *Saccharomyces, Kluveromyces*, or *Candida*, and a member of the species *Saccharomyces ceriviseae, Kluveromyces lactis*, or *Candida albicans*.

Regulatable promoters, particularly inducible promoters useful in the invention include, but are not limited to, P_{xylA} plus the *xylR* repressor gene, from various bacteria, such as *Bacillus* sp. and *Lactobacillus pentosus*; P_{lacA} plus the *lacR* repressor gene, from various bacteria, such as *E. coli* and *Lactococcus lacti*; hybrid promoters consisting of the *E. coli lac* repressor/operator and the –10 and –35 regions of various promoters, such as phages SP0-1 (known as P_{spac}) and T5; $P_{xyl/tet}$ - a hybrid consisting of the *E. coli* Tn*10 tet* repressor/operator and the *Bacillus subtilis xylA* –10 and –35 regions; P_{T7} plus the T7 RNA

polymerase gene under the control of one of the described promoters; P_{trp} from various bacteria; ϕ31 middle promoter from *Lactococcus lactis*; Lantibiotic inducible promoters, such as P_{nisA} or P_{nisF} from *Lactococcus lactis* or P_{spaB} from *Bacillus subtilis*; and Galactose-inducible and Thiostrepton-inducible promoters from *Streptomyces lividans*

In the absence of inducer for an inducible promoter (*e.g.*, xylose for P_{xylA}; IPTG for P_{spac}, etc.), low to negligible levels of RAT will be expressed. Thus, when glutamyl tRNA synthetase (ERS) mischarges $tRNA^{Gln}$ with Glu, the cell will no longer be able to convert the Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ by transamidation. In mammalian chloroplasts misacylated Glu-$tRNA^{Gln}$ has been shown to be rejected by EF-Tu, and is thus not brought to the ribosome and hence not utilized in protein synthesis (Stanzel-M et al. 1994 Eur. J. Biochem. 219, 435-439). As the cell now has no means of incorporating the amino acid glutamine into nascent proteins, translation will be aborted, leading to cell death. It is provided by the methods of the invention that an analogous will occur in bacteria.

In order to overcome the toxicity caused by ERS, a certain minimum level of RAT enzyme must be present in the cell. This level will relate to a certain level of inducer (herein "level 1"). By adding excess inducer, the levels of RAT will exceed the minimum levels required to relieve ERS toxicity (herein "level 2"). While not wanted to be limited by a mechanism, a schematic of this scenario is illustrated diagrammatically in Figure 2.

In a preferred embodiment, an antimicrobial compound screen may be run at both level 1 and level 2, and hits will be determined, for example, by their ability to cause cell death, as measured by a reduction in OD at 600nm, mimicing the effect of having no inducer of RAT expression present. These hits will include both RAT-specific inhibitors and general bactericidals. The following strategy may be used to differentiate between the two.

Hits that cause toxicity at RAT level 1 but not level 2 will be deemed to be RAT-specific inhibitors and not general bactericidals, on the basis that they are not potent enough to inhibit all of the excess of RAT present at level 2, and that general bactericidals will inhibit at both levels. However, not all hits that inhibit at both levels will be general bactericidals. RAT inhibitors that are particularly potent will work at both levels. Therefore, a further screen may be employed for hits in that category. This will involve rerunning the screen using a reduced concentration of these hits, to look for any that only cause toxicity at RAT level 1. These will be deemed to be RAT-specific inhibitors.

An alternative preferred format of this screen will use a different, more sensitive readout to OD reduction in order to assess hits. This will involve expressing luciferase (from the *luxAB* genes) in the cells in the presence of its octanal substrate, which will result in light production. This can be measured using a luminometer. Hits will be identified by their ability to reduce the light output.

Moreover, as *E.coli* belongs to the gamma subdivision of purple bacteria, it does not possess a RAT enzyme. Although neither of the two tRNA(Gln) species in *E.coli* is recognized by *Escherichia coli* (herein *E. coli*) ERS, one of them *is* recognised by glutamyl tRNA synthetase from *Bacillus subtilis* (herein *B. subtilis*). Applicants used these observations and others to form the basis for the present invention, since Applicants believe that the glutamyl tRNA synthetase enzyme from *S.aureus* will also misacylate the same tRNA(Gln) from *E.coli*; this seems likely as *S.aureus* has RAT activity and *S.aureus* glutamyl tRNA synthetase gene was difficult to clone in *E.coli*.

A method of screening for antimicrobial drugs comprising the steps of: providing at least one cell naturally lacking a RAT gene and comprising at least one recombinant glutamyl tRNA synthetase gene and at least one recombinant RAT gene; contacting the cell with at least one candidate compound; and detecting altered metabolism in the cell of the contacting step. As used herein "RAT gene" means any or all of the genes encoding RAT protein subunits in an enzymatically active or biologically functional RAT protein complex. As used herein "RAT protein" means any or all of the RAT protein subunits in an enzymatically active or biologically functional RAT protein complex.

One preferred embodiment of the invention provides the steps of: expressing *S.aureus* glutamyl tRNA synthetase and RAT, or a variant thereof, in *E.coli*. It is preferred that these gene be on separate plasmids configured such that RAT levels may be regulated. *E. coli* may be contacted with candidate compounds to determine whether they possess antimicrobial activity, and such antimicrobial activity of the candidate compound may be tested. Without being limited by a mechanism of action for the assays of the invention, it is believed that this *E. .coli* should not be harmed by the misacylated Glu-tRNA(Gln) as it should be corrected by RAT. Inhibition of RAT activity by a candidate compound possessing antimicrobial activity will result in accumulation of Glu-tRNA(Gln) and consequent cell death. This method has an advantage of being specific for *S.aureus* RAT in a cellular environment and selecting for compounds that display a capacity to penetrate to

the cytosol; achieving compound penetration into *E.coli* is likely to be more demanding than penetration into, e.g. *S.aureus*.

The determining step of the invention may be carried out in many ways in view of the teachings of the present invention. In the methods of the invention, the determining step may be performed using any method to detect altered translation or mysacylation. For example, altered translation is monitored by the introduction of a label into the amino acids of the protein. As a further example, the skilled artisan may detect altered translation directly, such as at the protein level, or indirectly, such as by detecting alterations in the activity of the protein.

Another embodiment of the determining step provides detecting Gln-tRNA(Gln) starvation in the host cell following contacting such cell with at least one candidate compound.

Methods are provided herein wherein the determining step comprises detecting or measuring any aspect of altered cellular metabolism, such as detecting or measuring the inhibition of RAT protein activity or RAT gene expression. The determining step may further comprises detecting a toxic accumulation of Glu-tRNA(Gln) and/or cell death.

Following contacting the host cell with at least one candidate compound, if it is determined that there is altered translation, Gln-tRNA(Gln) starvation, cell death, or any other alteration of host cell metabolism detected as provided herein, the candidate compound may be useful as an antimicrobial compound. This may be readily determined using any of the many well known methods for testing antimicrobial activity, such as, for example, by disk diffusion assay followed by an MIC determination.

Preferred method comprise recombinant glutamyl tRNA synthetase and RAT genes that are on an episomal element or integrated into a chromosome of the host cell. It is particularly preferred that in such methods that RAT gene expression level is regulated or regulatable.

The RAT gene, or variants thereof, in the compositions and methods of the invention may be obtained from any source. Methods of the invention may comprise a RAT gene selected from the group consisting of a Gram positive bacterium, a Gram negative bacterium, a streptococcus, S. pneumoniae, a staphylococcus, S. aureus, enterococci, Enterococcus faecalis, Enterococcus faecium, a Bacillus, and *Bacillus subtilis*, among other bacteria.

Compositions and methods of the invention may comprise glutamyl tRNA synthetase gene, or variants thereof, selected from the group consisting of a Gram positive

bacterium, a Gram negative bacterium, a streptococcus, S. pneumoniae, a staphylococcus, S. aureus, enterococci, Enterococcus faecalis, Enterococcus faecium, a Bacillus, and *Bacillus subtilis*, among other bacteria.

Compositions and methods of the invention may a cell possessing a glutaminyl tRNA synthetase or lacking a RAT gene. Also provided by the invention in an isolated bacterial cell lacking a RAT gene and/or comprising at least one recombinant bacterial glutamyl tRNA synthetase gene and at least one recombinant bacterial RAT gene. These glutamyl tRNA synthetase and/or RAT genes may be present on episomal element or integrated into a chromosome of the host cell. Host cells also provided herein comprise a glutamyl tRNA synthetase gene from *S. aureus*. Host cells are provided possessing a glutaminyl tRNA synthetase and/or lacking a RAT gene.

A preferred embodiment provides a method wherein the polypeptide translated in the screening assay is a nascent polypeptide chain or a substantially purified polypeptide. In any case, the polypeptide in the assay may be labeled using any of the well known polypeptide labeling methods, such as, radiolabeling or chromogenic labeling, or detected using an appropriate antibody and immunopreciptation reaction. It is preferred that the label used be readily observable or detectable, such as by being luminescent, radiolabeled, colored or fluorescent.

Another preferred embodiment provides a method wherein a test protein is isolated, such as being prepared to a substantially purified form. Test proteins may be isolated using methods well known in the art, including, for example, density centrifugation or gel electrophoresis.

Yet another preferred embodiment provides a method wherein a RAT gene or protein is selected from the group consisting of a Gram positive bacterium, a Gram negative bacterium, a streptococcus, S. pneumoniae, a staphylococcus, S. aureus, enterococci, Enterococcus faecalis, Enterococcus faecium, a Bacillus, and *Bacillus subtilis*.

A still more preferred embodiment provides a method wherein a RAT gene or protein is derived or isolated from *S. aureus*. Examples of RAT genes and proteins derived or isolated from *S. aureus* are provided in Table 1.

TABLE 1

RAT genes and proteins

Polynucleotide sequence of ratA [SEQ ID NO:1]

5'-
ATGAGCATTCGCTACGAATCGGTTGAGAATTTATTAACTTTAATAAAAGACAAAAAAATCAAACCATCTG
ATGTTGTTAAAGATATATATGATGCAATTGAAGAGACTGATCCAACAATTAAGTCTTTTCTAGCGCTGGA
TAAAGAAAATGCAATCAAAAAAGCGCAAGAATTGGATGAATTACAAGCAAAAGATCAAATGGATGGCAAA
TTATTTGGTATTCCAATGGGTATAAAAGATAACATTATTACAAACGGATTAGAAACAACATGTGCAAGTA
AAATGTTAGAAGGTTTTGTGCCAATTTACGAATCTACTGTAATGGAAAAACTACATAAAGAGAATGCCGT
TTTAATCGGTAAATTAAATATGGATGAGTTTGCAATGGGTGGTTCAACAGAAACATCTTATTTCAAAAAA
ACAGTTAACCCATTTGACCATAAAGCAGTACCAGGTGGTTCATCAGGTGGATCTGCAGCAGCAGTTGCAG
CTGGCTTAGTACCATTTAGCTTAGGTTCAGACACAGGTGGTTCAATTAGACAACCGGCTGCATATTGTGG
CGTTGTCGGTATGAAACCAACATACGGTCGTGTATCTCGATTTGGATTAGTTGCTTTTGCATCTTCATTA
GACCAAATTGGTCCATTGACTCGAAATGTAAAAGATAATGCAATCGTATTAGAAGCTATTTCTGGTGCAG
ATGTTAATGACTCTACAAGTGCACCAGTTGATGATGTAGACTTTACATCTGAAATTGGTAAAGATATTAA
AGGATTAAAAGTTGCATTACCTAAAGAATACTTAGGTGAAGGTGTAGCTGATGACGTAAAAGAAGCAGTT
CAAAACGCTGTAGAAACTTTAAAATCTTTAGGTGCTGTCGTTGAGGAAGTATCATTGCCAAATACTAAAT
TTGGTATTCCATCATATTACGTGATTGCATCATCAGAAGCTTCGTCAAACCTTTCTCGTTTTGACGGAAT
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Polypeptide sequence of ratA [SEQ ID NO:2] deduced from the sequence of SEQ ID NO:1

NH_2-
MSIRYESVENLLTLIKDKKIKPSDVVKDIYDAIEETDPTIKSFLALDKENAIKKAQELDELQAKDQMDGK
LFGIPPMGIKDNILITNGLETTCASKMLEGFVPIYESTVMEKLHKENAVLIGKLNMDEFAMGGSTETSYFKK
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FGKEVKRRIFLGTFALSSGYYDAYYKKSQKVRTLIKNDFDKVFENYDVVVGPTAPTTAFNLGEEIDDPLT
MYANDLLTTPVNLAGLPGISVPCGQSNGRPIGLQFIGKPFDEKTLYRVAYQYETQYNLHDVYEKL-COOH

Polynucleotide sequence of rat B [SEQ ID NO:3]

5'-
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TGGGCTTCTTAGTTGGTCAAATTATGAAAGCGTCTAAAGGTCAAGCTAATCCACAATTAGTAAATCAACT
ATTAAAACAAGAATTAGATAAAAGA-3'

Polypeptide sequence of rat B [SEQ ID NO:4] deduced from the sequence of SEQ ID NO:3.

NH_2-
MHFETVIGLEVHVELKTDSKMFSPSPAHFGAEPNSNTNVIDLAYPGVLPVVNKRAVDWAMRAAMALNMEI
ATESKFDRKNYFYPDNPKAYQISQFDQPIGENGYIDIEVDGETKRIGITRLHMEEDAGKSTHKGEYSLVD
LNRQGTPLIEIVSEPDIRSPKEAYAYLEKLRSIIQYTGVSDVKMEEGSLRCDANISLRPYGQEKFGTKAE
LKNLNSFNYVRKGLEYEEKRQEEELLNGGEIGQETRRFDESTGKTILMRVKEGSDDYRYFPEPDIVPLYI
DDAWKERVRQTIPELPDERKAKYVNELGLPAYDAHVLTLTKEMSDFFESTIEHGADVKLTSNWLMGGVNE
YLNKNQVELLDTKLTPENLAGMIKLIEDGTMSSKIAKKVFPELAAKGGNAKQIMEDNGLVQISDEATLLK
FVNEALDNNEQSVEDYKNGKGKAMGFLVGQIMKASKGQANPQLVNQLLKQELDKR-COOH

Sequences from *Staphylococcus aureus* ratC polynucleotide sequence [SEQ ID NO:5].

5'-
ATGACAAAAGTAACACGTGAAGAAGTTGAGCATATCGCGAATCTTGCAAGACTTCAAATTTCTCCTGAAG
AAACGGAAGAAATGGCCAACACATTAGAAAGCATTTTAGATTTTGCAAAACAAAATGATAGCGCTGATAC
AGAAGGCGTTGAACCTACATATCACGTTTTAGATTTACAAAACGTTTTACGTGAAGATAAAGCAATTAAA
GGTATTCCGCAAGAATTAGCTTTGAAAAATGCCAAAGAAACAGAAGATGGACAATTTAAAGTGCCTACAA
TCATGAATGAGGAGGACGCG-3'

ratC polypeptide sequence [SEQ ID NO:6] deduced fr m the polynucleotide sequence in this table [SEQ ID NO:5].

NH_2 -
MTKVTREEVEHIANLARLQISPEETEEMANTLESILDFAKQNDSADTEGVEPTYHVLDLQNVLREDKAIKG
IPQELALKNAKETEDGQFKVPTIMNEEDA-COOH

Any organism possessing a glutaminyl-tRNA synthetase activity or not possessing a RAT enzyme, such the gamma subdivision of purple bacteria may be engineered to be useful with certain methods of the invention using a heterogeneous RAT gene and glutamyl-tRNA synthetase (ERS) transformed into such organism. For example, organisms or host cells useful in the methods of the invention include organisms and host cells selected from the group consisting of a eubacterium, an archaebacterium, gamma subdivision of the purple bacteria, a eukaryote, including lower eukaryotes, such as fungi, protozoa, and cells from higher eukaryotes, such as mammalian cells, CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells.

Preferred organisms useful as host cells in the methods of the invention include those which do not possess a RAT enzyme and for which a tRNA(Gln) from such organism is recognised by a heterologous glutamyl tRNA synthetase, such as a glutamyl tRNA synthetase from another species. Applicants used these observations

Once the composition or cells comprising a RAT gene as described in the forgoing is prepared, one or more candidate compounds are added to the composition to test whether any of the candidate compounds is associated with altered translation. If a mixture of candidate compounds is associated with an alteration of RAT expression or activity or glutamyl tRNA synthetase expression or acivity, the mixture may be deconvoluted to determine which compound or compounds is active. One method to achieve this is to test each component of the mixture separately in the assay. Deconvolution may also be performed using any of the known deconvolution methods.

It is preferred that the method of the invention is formatted for high throughput screening (herein "HTS"). Skilled artisans can readily adapt the method of the invention for HTS. A particularly preferred embodiment of the screening methods of the invention is a high throughput screen for compounds that interfere with the proper functioning of RAT gene expression or protein and/or glutamyl tRNA synthetase gene expression or protein, such as compounds that are associated with altered translation.

Potential antimicrobial compounds identified using the method of the invention include, among other things, small organic molecules, polynucleotides, peptides, polypeptides and antibodies that bind RAT polynucleotides or polypeptides, or mimic the activity of a RAT polypeptides.

Potential antagonists include a small molecule that binds to RAT polynucleotides or polypeptides thereby preventing binding of natural factors, such as, for example, tRNAs, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules.

The invention further provides assay packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Examples of preferred kits are kits comprising at least one bacterial cell lacking a RAT gene and the cell comprising at least one bacterial glutamyl tRNA synthetase gene and at least one bacterial RAT gene. A further preferred kit comprises a polynucleotide encoding a RAT gene and a polynucleotide encoding a glutamyl tRNA synthetase gene. Kits comprising a RAT gene expressibly linked to an inducible promoter are also preferred.

Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

What is claimed is:

1. A method of screening for antimicrobial drugs comprising the steps of: providing at least one cell naturally lacking a RAT gene and comprising at least one recombinant or wild type glutamyl tRNA synthetase gene and at least one recombinant RAT gene; contacting said cell with at least one candidate compound; and detecting altered metabolism in said cell of the contacting step.

2. The method of claim 1 wherein the recombinant glutamyl tRNA synthetaseand RAT genes are on an episomal element or integrated into a chromosome of said cell.

3. The method of claim 2 wherein RAT gene expression level is regulated.

4. The method of claim 1 wherein at least one RAT gene is a RAT gene selected from the group consisting of a Gram positive bacterium, a Gram negative bacterium, a streptococcus, *S. pneumoniae*, a staphylococcus, *S. aureus*, enterococci, *Enterococcus faecalis, Enterococcus faecium*, a Bacillus, and *Bacillus subtilis*.

5. The method of claim 1 wherein the glutamyl tRNA synthetase gene is a glutamyl tRNA synthetase gene selected from the group consisting of a Gram positive bacterium, a Gram negative bacterium, a streptococcus, *S. pneumoniae*, a staphylococcus, *S. aureus*, enterococci, *Enterococcus faecalis, Enterococcus faecium*, a Bacillus, and *Bacillus subtilis*.

6. The method of claim 1 wherein said altered metabolism comprises inhibition of RAT protein activity.

7. The method of claim 1 wherein said detecting step further comprises detecting a toxic accumulation of Glu-tRNA(Gln) or toxic incorporation of a glutamyl residue in place of a glutaminyl residue in nascent protein chains.

8. The method of claim 1 wherein said detecting step further comprises detecting cell death or a reduction in growth rate or amount.

9 The method of claim 1 wherein said cell possesses or lacks a glutaminyl tRNA synthetase or possesses or lacks a RAT gene.

10. An isolated bacterial cell lacking a RAT gene and comprising at least one recombinant or wild type bacterial glutamyl tRNA synthetase gene and at least one recombinant bacterial RAT gene.

11. The cell of claim 10 wherein said glutamyl tRNA synthetase and RAT genes are on episomal element or integrated into a chromosome of said cell.

12. The cell of claim 10 wherein said RAT gene expression level is regulated.

13. The cell of claim 10 wherein said RAT gene is a *S. aureus* RAT gene.

14. The cell of claim 10 wherein said glutamyl tRNA synthetase gene is a *S. aureus* glutamyl tRNA synthetase gene.

15. The cell of claim 10 wherein said cell possesses a glutaminyl tRNA synthetase or lacks a RAT gene.

16. The method of claim 1 wherein said detecting step further comprises detecting altered translation.

17. The method of claim 1 wherein said detecting step further comprises detecting altered test protein.

18. A kit comprising at least one bacterial cell lacking a RAT gene and said cell comprising at least one bacterial glutamyl tRNA synthetase gene and at least one bacterial RAT gene.

19. A kit comprising a polynucleotide encoding a RAT gene and a polynucleotide encoding a glutamyl tRNA synthetase gene.

20. A polynucleotide comprising a RAT expressibly linked to an inducible promoter.

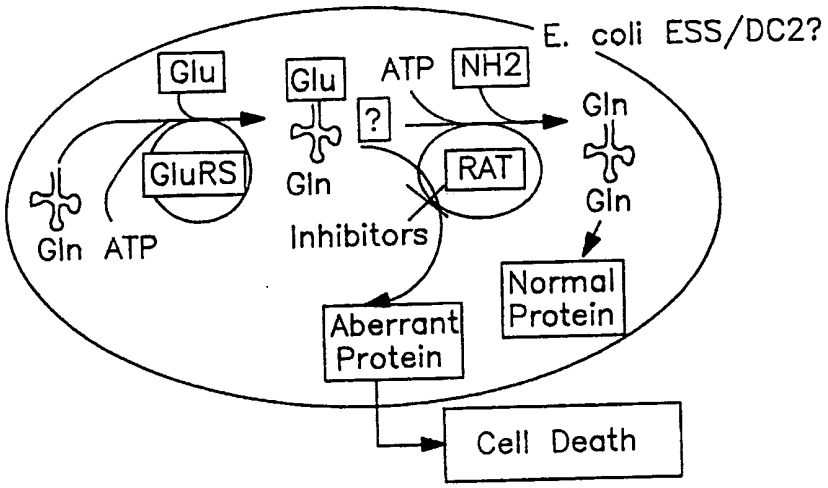

E. coli ESS/DC2?
Glu
Glu
ATP
NH2
?
Gln
GluRS
Gln
RAT
Gln
Gln
ATP
Inhibitors
Normal Protein
Aberrant Protein
Cell Death

FIG. 1

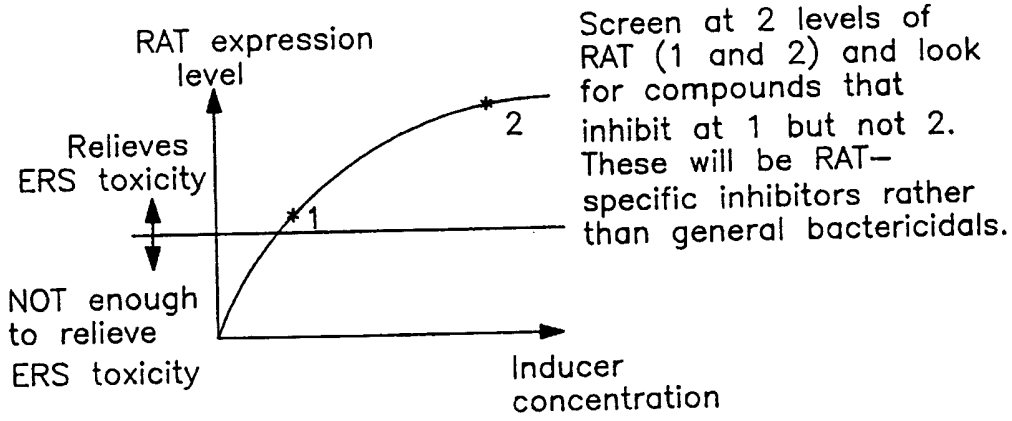
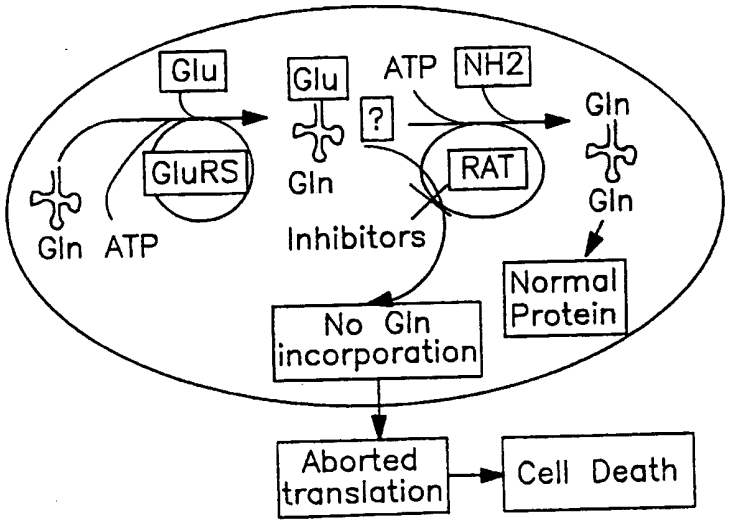

RAT expression level
Relieves ERS toxicity
NOT enough to relieve ERS toxicity
1
2
Inducer concentration
Screen at 2 levels of RAT (1 and 2) and look for compounds that inhibit at 1 but not 2. These will be RAT-specific inhibitors rather than general bactericidals.

FIG. 2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Glu
ATP
NH2
?
Gln
GluRS
Gln
RAT
Gln
Gln
ATP
Inhibitors
Normal Protein
No Gln incorporation
Aborted translation
Cell Death

FIG. 3

SEQUENCE LISTING

<110> Michael T. Black
Howard Kallender

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<141> October 2, 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20582

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q1/68; C12N 15/00
US CL :435/172.3, 6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: rat gene, transferase?, trna, synthetase?, misaminoacylat?, glutamyl?, glu-trna, gln (2a) trna

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,643,722 A (ROTHSCHILD et al) 01 July 1997, see title, abstract and claims, and entire document.	1-20
A	US 5,646,024 A (LEEMANS et al) 08 July 1997, see title, abstract, claims and entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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